Natural colonization and adaptation of a mosquito species in Galápagos and its implications for disease threats to endemic wildlife

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Emerging infectious diseases of wildlife have been recognized as a major threat to global biodiversity. Endemic species on isolated oceanic islands, such as the Galápagos, are particularly at risk in the face of introduced pathogens and disease vectors. The black salt-marsh mosquito (*Aedes taeniorhynchus*) is the only mosquito widely distributed across the Galápagos Archipelago. Here we show that this mosquito naturally colonized the Galápagos before the arrival of man, and since then it has evolved to represent a distinct evolutionary unit and has adapted to habitats unusual for its coastal progenitor. We also present evidence that *A. taeniorhynchus* feeds on reptiles in Galápagos in addition to previously reported mammal and bird hosts, highlighting the important role this mosquito might play as a bridge-vector in the transmission and spread of extant and newly introduced diseases in the Galápagos Islands. These findings are particularly pertinent for West Nile virus, which can cause significant morbidity and mortality in mammals (including humans), birds, and reptiles, and which recently has spread from an introductory focus in New York to much of the North and South American mainland and could soon reach the Galápagos Islands. Unlike Hawaii, there are likely to be no highland refugia free from invading mosquito-borne diseases in Galápagos, suggesting bleak outcomes to possible future pathogen introduction events.

Aedes | disease vector | phylogenetics | West Nile virus

Emerging infectious diseases of wildlife have been recognized as a major threat to global biodiversity (1). One important driver of disease emergence is the introduction of vector-borne pathogens into previously unexposed areas, largely due to globalization (increased movement of humans and resources around the earth) and human-induced ecological changes (2). Endemic species in isolated oceanic islands are particularly at risk in the face of introduced pathogens and disease vectors; a classic example of this phenomenon is the dramatic decline of Hawaiian forest birds caused by the introduction of avian malaria, avianpoxvirus, and mosquito vectors into the islands (3, 4). The Galápagos Islands form one of the most pristine archipelagos on Earth, with much of their endemic fauna still intact, and they provide an exceptional demonstration of evolutionary processes (5). Unfortunately, growing tourism and population pressure have led many endemic taxa to decline, mainly due to habitat destruction, overexploitation, and the introduction of invasive species (6, 7). Recently, the introduction of new pathogens and disease vectors has also been recognized as a major threat to Galápagos Island biodiversity (8–11), underlining the need to understand the processes by which novel vector-borne pathogens emerge and spread into new hosts or geographic ranges.

Mosquitoes are important disease vectors and have been implicated in the spread and establishment of novel pathogens on islands (3, 4). Only 3 mosquito species (Diptera: Culicidae) have been found in the Galápagos archipelago to date: The southern house mosquito (*Culex quinquefasciatus* Say), the black salt marsh mosquito (*Aedes taeniorhynchus* Wiedemann), and the globally distributed Yellow Fever mosquito (*Aedes aegypti* L.) (12). *Aedes aegypti*, introduced in the 1990s, is highly anthropophilic and is found only in urban zones on Santa Cruz island; therefore *C. quinquefasciatus* and *A. taeniorhynchus* are the only 2 mosquito species which might play a significant role in the transmission of wildlife diseases in the Galápagos Islands (8).

Culex quinquefasciatus, an important vector of wildlife diseases (e.g., avian malaria, avian pox), was introduced into Galápagos in 1985 and its presence is currently restricted to human settlements (10). In contrast, *A. taeniorhynchus* is widely distributed and thrives throughout the archipelago, sometimes constituting an important nuisance to wildlife, e.g., nesting birds (13). It is a brackish flood-water specialist found in temperate and tropical coastal areas of the Americas, from New Hampshire to Brazil on the Atlantic coast and from California to northern Peru on the Pacific coast (14). Its presence in the Galápagos Islands was first recorded in the late 1880s (15), but it is not known whether it arrived naturally or with man. *Aedes taeniorhynchus* in Galápagos has never been studied in depth and its potential importance as a disease vector has only recently been considered (8, 10). Elsewhere, few studies have focused on the black salt-marsh mosquito, and it is usually considered only as a nuisance mosquito with respect to human health. However, *A. taeniorhynchus* plays a major role in the transmission of the dog heartworm (*Dirofilaria immitis*) in South and Central America (16) and has been identified as a competent vector of many arthropod-borne viruses such as St. Louis encephalitis virus and West Nile virus (WNV) (17, 18). *Aedes taeniorhynchus* has been considered as an important bridge-vector of WNV between birds and mammalian hosts despite its relatively low susceptibility to infection under experimental conditions (17, 19).

If the presence of *A. taeniorhynchus* in Galápagos is indeed the result of an early natural colonization, then in the absence of other mosquitoes it might have successfully adapted and spread

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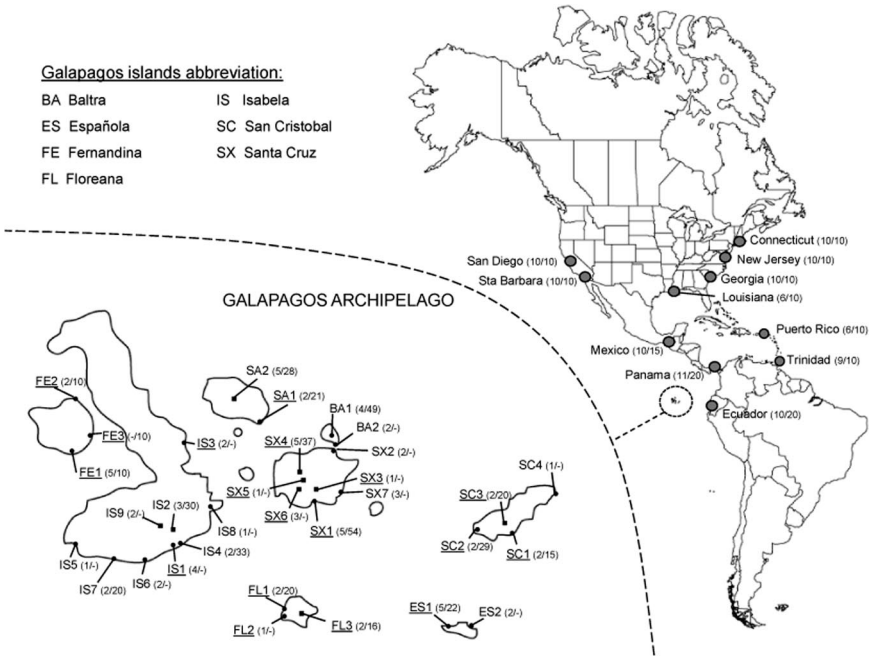

Fig. 1. Map of the sites where *Aedes taeniorhynchus* specimens were collected on the American continents and on the Galápagos Islands. Numbers between brackets are the number of specimens collected in each site used for the mtDNA and microsatellite studies, respectively. Sites where bloodmeals from bloodfed mosquitoes were analyzed are underlined.

into the different available environments in the islands. Thus, its ecology and behavior could have significantly diverged from the continental source population, and it is likely to play a role in the ecology of vector-borne wildlife diseases in the archipelago. Here we employ a phylogenetic approach by using mitochondrial DNA (mtDNA) sequence analysis and microsatellite genotyping to determine the origins and demographic history of this species in the islands, and to explore whether genetic differentiation and local adaptations of *A. taeniorhynchus* specific to the Galápagos environment have occurred. In addition, mtDNA analysis of mosquito bloodmeals was undertaken to gain insight into the host feeding range of *A. taeniorhynchus* in the archipelago. Implications for the epidemiology of endemic and introduced vector-borne pathogens in the endemic fauna are discussed.

Results and Discussion

Phylogeny of *A. taeniorhynchus*: Colonization of Galápagos Islands. We analyzed a combined sequence dataset of portions of the NADH dehydrogenase subunit 5 (*ND5*) and the cytochrome oxydase subunit 2 (*COII*) genes, with a total length of 1,242 bp, obtained from mosquitoes collected in various islands and habitat zones of the Galápagos Archipelago (78 specimens) and in countries on the American continents spanning the species' geographic range (112 specimens, see Fig. 1). A total of 68 variable sites defined 31 and 62 individual sequences (haplotypes) in the Galápagos Islands and the American continents, respectively (EMBL accession numbers FM992116–FM992321). Both the highest haplotype diversity and the highest nucleotide diversity within the Galápagos Islands were found in Isabela, the largest island of the archipelago, whereas on the continents, the mosquitoes sampled from the Atlantic coast were characterized by the highest mtDNA diversity (see Table S1).

We inferred the phylogenetic relationships between each of the Galápagos and continental haplotypes by using maximum likelihood and Bayesian inference methods. Phylogenies group the Galápagos haplotypes in a monophyletic clade separated from continental haplotypes with a bootstrap support value of 68% (maximum likelihood) and a posterior probability of 100% (Bayesian inference, Fig. 2*A*). The trees also supported the separation of the continental haplotypes into Pacific U.S. coast and Atlantic U.S. coast clusters. South American haplotypes grouped with the Atlantic coast cluster, whereas haplotypes found in Central America were distributed between the 2 clusters. The Bayesian tree suggested an additional Central American cluster containing most of the Central American haplotypes that were grouped with the Atlantic coast cluster in the maximum likelihood tree (Fig. 2*A*). These results suggest that populations in the mainland are characterized by a complex biogeographic history, with the presence of 1 main lineage distributed along the Atlantic coast of the U.S. and throughout Latin America and another lineage isolated in the Pacific coast of the U.S. with secondary contact between the 2 lineages in Central America. The phylogenetic trees also show that the Galápagos clade is most closely related to the U.S. Atlantic coast–Latin American cluster, suggesting that the Galápagos population originated from this main *A. taeniorhynchus* lineage.

A signature of colonization followed by demographic expansion of the Galápagos population across the archipelago was found by using tests for polymorphism (Fu's Fs, Tajima's D), mismatch distribution analysis, and a median-joining haplotype network (see Table S2 and Figs. S1 and S2). We used 12 microsatellite markers to genotype 423 Galápagos samples and 135 Continental samples to confirm the results obtained with the mtDNA markers (Fig. 1). Allele-sharing and Cavalli-Sforza distance trees constructed with the microsatellite data showed results very similar to the mtDNA sequence analysis, and unambiguously supported a Latin American origin of the Galápagos clade (Fig. 2*B*). Altogether, the analysis of nuclear and mitochondrial markers demonstrate that the Galápagos population of *A. taeniorhynchus* originated from a single colonization event and now represents a distinct evolutionary unit clearly divergent from the continental populations.

We estimated that the Galápagos and continental clades diverged 200,000 years ago (95% confidence intervals: 100,000–350,000 years). Due to the lack of fossil calibration data, we based our calculations on a rate of nucleotide substitution of

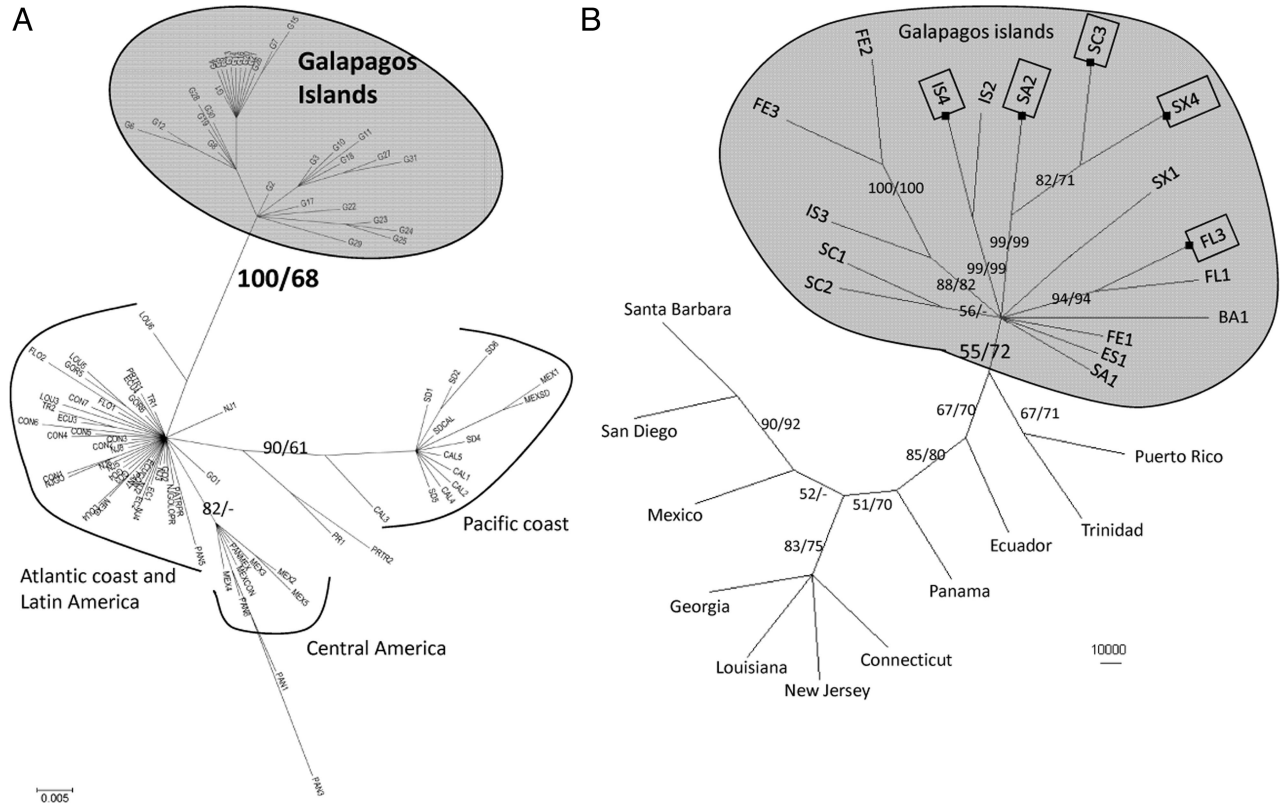

Fig. 2. Unrooted Bayesian tree based on combined COII and ND5 mtDNA gene datasets (*A*) and unrooted distance tree based on proportion of shared alleles (*B*), showing the relationships between *A. taeniorhynchus* populations. Haplotype and population name codes refer to names given in Fig. 1 and Table S1. Numbers beside branches indicate supports for the nodes of the trees from posterior probability/bootstrap values (> 50%) obtained with Bayesian inference and maximum likelihood methods, respectively (*A*), and bootstrap values (>50%) from shared allele and Cavalli-Sforza distance calculations, respectively (*B*). The Galápagos cluster is highlighted by a gray circle. Highland populations are surrounded by a rectangle in *B*.

1.15% per million years, a rate widely used for insect mtDNA genes (20). Although imprecise, this estimation demonstrates that the colonization by *A. taeniorhynchus* was not human-driven, unlike the 2 other mosquito species present on the archipelago, because confidence intervals derived from a relaxed Bayesian molecular clock (which allows for some variation in mutation rate) fall >99,000 years before the archipelago's discovery by humans. Actual mutation rates would need to be between 200 and 1,000 times higher, respectively, to push the confidence intervals into the period of first discovery (≈1535 A.D.) or colonization (early 20th century) by humans. In contrast, colonization by other endemic insects, some of which arrived soon after the archipelago was formed, was ancient (>5 million years ago) (21). This relatively recent colonization of *A. taeniorhynchus* compared with other Galápagos endemic fauna suggests that its arrival into a system with no other mosquito disease vectors may have precipitated alterations of the dynamics for existing endemic vector-borne pathogens or allowed novel diseases to invade, signatures of which might still be discernible today in both hosts and pathogens.

Adaptation of *A. taeniorhynchus* to the Galápagos Environment. Our estimation of the time since divergence between the Galápagos and continental populations indicates that *A. taeniorhynchus* in the Galápagos might have had time to adapt to the exceptional environment of the archipelago and the empty niches it would have found there. On the continent, the species is rarely found >6 km from the coast and has been reported to breed inland only exceptionally (22–24). In the Galápagos Archipelago, we have regularly caught *A. taeniorhynchus* in the humid highland zone of various islands up to 20 km from the coast and at 700-m altitude. Twenty-four specimens sampled from 8 highland sites in the islands of Floreana, Isabela, San Cristobal, Santa Cruz, and Santiago were incorporated in the mtDNA study, and 131 highland specimens from the same islands were genotyped using the microsatellite markers (Fig. 1). Sixty-seven percent of the highland samples were characterized by unique mtDNA haplotypes not present on the coast (Table S1). Phylogenetic trees constructed with the microsatellite data grouped 3 highland populations from 3 different islands in one cluster (SA2, SC3, and SX4 in Fig. 2*B*), separated from the coastal populations of their own island. In addition, individual and population clustering tests performed on the microsatellite data also clearly showed a strong genetic similarity between the same 3 highland populations, whereas coastal populations of these islands and Baltra Island are clustered together (Fig. 3 *A* and *B*). The 3 highland populations show significant genetic differentiation in terms of pairwise F_{ST} comparisons among themselves (0.03 to 0.06, $P < 0.001$), although the values obtained were lower than F_{ST} values for comparisons among coastal and highland populations (0.06 to 0.19, $P < 0.001$; Table S3). The F_{ST} value of 0.19 for the comparison between the coastal and highland populations of Santa Cruz Island (14 km apart) is relatively large and corresponds to the range of values normally obtained for mosquito populations separated by larger geographical distances (25). The consistent differentiation between coastal and highland populations supports the hypothesis that selection may play a role in maintaining these genetic differences, and warrants further investigation.

These results suggest a single colonization event in the past,

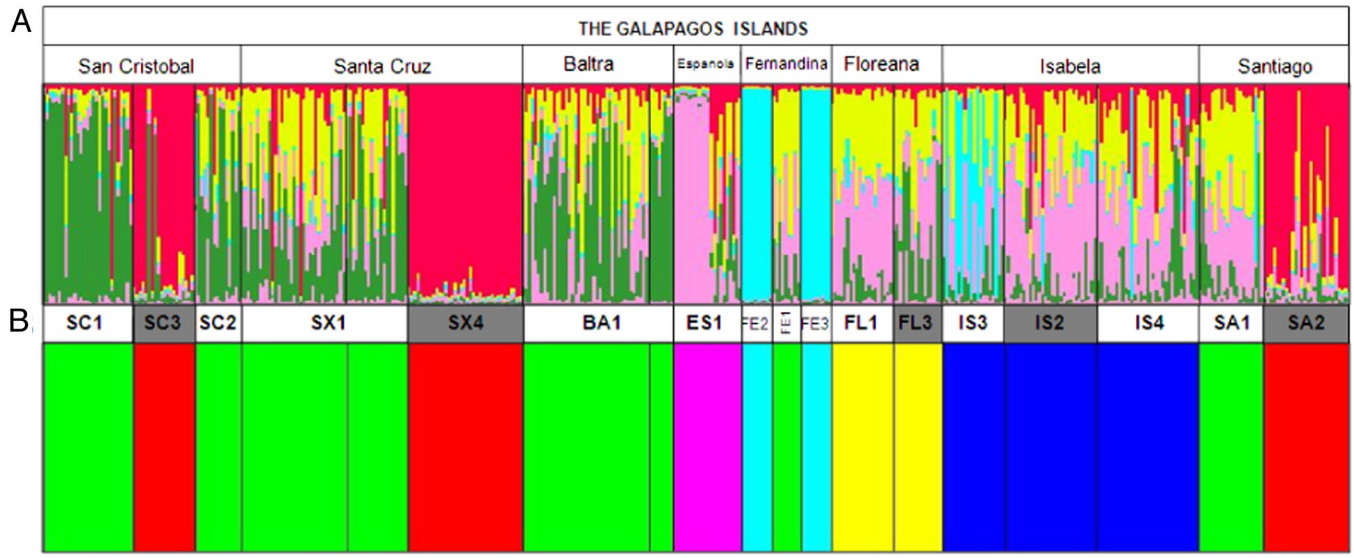

Fig. 3. Results of Bayesian individual clustering for the Galápagos microsatellite dataset (*A*), and of Bayesian population clustering for the Galápagos dataset (*B*). In both *A* and *B*, individuals are grouped by sampling location (or geographical entities) within each island. Abbreviations shown between the 2 panels are code names of sampling locations referring to codes given in Fig. 1. (*A*) Each individual is represented by a vertical bar partitioned into colored segments according to the probability of belonging to one of the *K*-color-coded genetic clusters, *K* being defined as the number of clusters that best fit with our data (here $K = 6$, identified by the 6 colors in the graph). (*B*) In the population clustering, the sampling locations/geographical entities are grouped by color to indicate which groups are likely to represent distinct populations. Highland populations are indicated with labels highlighted in gray.

from the coastal to the highland environment, with subsequent migrations of breeding highland populations between islands of the archipelago. This event might have been facilitated by a lack of competition from other mosquito species, but genetic adaptation appears to have been a requisite for such inland colonization to occur. Reports from the early work of Belkin and collaborators (26), identified *A. taeniorhynchus* breeding in bromeliads in the highland forests of Santa Cruz Island. Together with our genetic data, this evidence suggests that since the colonization of the archipelago, *A. taeniorhynchus* may have not only spread to occupy new areas free of competitors, but also appears to have radiated and adapted to different ecological niches in the archipelago.

The remaining highland mtDNA haplotypes belonged to the most frequent coastal haplotypes (Table S1 and Fig. S2), indicating that some migration from the coast to the highlands may still occur, although with limited breeding success. This hypothesis is supported by the identification of 7 potential migrants between coastal and highland populations using a Bayesian assignment method on the microsatellite data. The genetic similarity of mosquito populations in the islands of Santa Cruz, San Cristobal, Santiago, and Baltra (Fig. 3 *A* and *B*) also suggests ongoing movement of mosquitoes between these islands. These movements could happen naturally when moist conditions and winds are favorable, such as during El Niño events (27), although the distance between San Cristobal and Santa Cruz (≈80 km) would make such events sporadic. This pattern of migration could also be linked with human activities, because 3 of these islands are inhabited, are connected by intense boat traffic, and receive cargo boats and planes from the mainland. A more detailed population genetic study using microsatellite loci on mosquitoes collected at different temporal and geographical locations within each island should give further insight to the migration patterns between ecological zones and between islands, and should help determine whether mosquitoes are being transported from the mainland into the Galápagos Islands.

Previously, *A. taeniorhynchus* had been shown to feed principally on large mammals and occasionally on birds (13, 28). Because the Galápagos Islands were depauperate in mammal species before the arrival of man, *A. taeniorhynchus* would have experienced pressure to broaden its host feeding range after it colonized the archipelago. We collected bloodfed mosquitoes in 6 highland and 12 coastal sites across 7 islands, including sites with different degrees of human disturbance (Fig. 1). A total of 105 bloodmeals were successfully analyzed using a PCR method targeting the cytochrome *b* (*cytb*) gene of vertebrates (29). Fifty-eight percent of the mosquitoes had fed on reptile blood, 47% on marine iguanas (*Amblyrhynchus cristatus*), and 11% on Galápagos tortoises (*Geochelone nigra*). Forty-one percent of the mosquitoes had fed on mammal blood [16% on Galápagos sea lions (*Zalophus wollebaeki*), 16% on human beings, and 9% on domestic animals]. Only 1 bloodmeal was identified as avian (flightless cormorant, *Phalacrocorax harrisii*). To our knowledge, this is the first time that *A. taeniorhynchus* has been found to feed on reptiles. Reptile bloodmeals were found across all of the islands sampled and across most environments, including a site in Espanola Island where birds were more abundant than reptiles, suggesting that reptile blood may have become a preferential choice for this mosquito. This observation may be a sign of adaptive divergent behavior of the Galápagos form of *A. taeniorhynchus*.

Implications for *A. taeniorhynchus* and Its Role as Vector of Wildlife Diseases in the Galápagos Islands. The genetic distance separating the Galápagos lineage from the most closely related continental lineage is of the order used to define species designations for other taxa, although no standardized DNA sequence difference exists for assessing insect species boundaries (30). Nevertheless, our phylogenetic results using both mitochondrial and nuclear DNA microsatellite markers, in combination with the other evidence of divergence presented in this paper, suggest that consideration should be given to reexamining the classification of *A. taeniorhynchus* in the Galápagos Islands. Mainland populations are known to show considerable morphological variation (26), and a more complete study of the taxonomic status of the whole *taeniorhynchus* complex across its range would be necessary to undertake this reclassification.

This study has important implications both for the population

dynamics of the Galápagos form of *A. taeniorhynchus* and for the epidemiology of vector-borne diseases in the archipelago. In addition to ranging across the archipelago, there have been highland colonizations by this mosquito, expanding the range of host populations and species that could be exposed to vector-borne diseases and greatly increasing the likelihood of the spread and establishment of introduced pathogens. Also, we have shown that *A. taeniorhynchus* feeds on Galápagos reptiles in addition to mammal and bird hosts, as previously reported for the mainland. Such diverse feeding behavior provides the potential for this mosquito to act as a bridge-vector across the majority of the Galápagos endemic wildlife. It is already known from studies on the mainland that *A. taeniorhynchus* transmits arboviruses, such as WNV, and other vector-borne pathogens (e.g., filarial nematodes and apicomplexan parasites); therefore, it is likely that this mosquito plays an important role in parasite transmission dynamics within the Galápagos. Crucially, because of its widespread distribution and diverse host range, *A. taeniorhynchus* should be considered key to the spread and establishment of novel, mosquito-borne pathogens, should these pathogens reach the archipelago. The risk is particularly high for pathogens such as WNV, which has a wide host range (including mammals, birds, and reptiles) and which probably constitutes the most concerning arbovirus threat to Galapagos vertebrates (8); it is predicted to reach the archipelago within a matter of years in the absence of mitigation measures (8). Due to the distribution of *A. taeniorhynchus* in Galápagos, unlike Hawaii (3, 4), there may not be highland refugia free from invading diseases, leading to a bleak outcome for endemic vertebrates, should an invasion occur. The impact of disease introduction to Galápagos could be heightened if human-aided movement of mosquitoes between the islands occurs, as suggested by our results. Monitoring of mosquito populations and strict adherence of disinsection protocols for both boats and planes must be implemented to reduce movement of mosquitoes to and among the Galápagos Islands to lower the risk of novel disease-spread across the archipelago. Galápagos *A. taeniorhynchus* appears to represent a striking example of adaptive diversification of a disease vector into novel environments and our findings demonstrate that this type of study on often-overlooked vectors is necessary to predict the full impact of pathogens invading new areas.

Materials and Methods

Sample Collection. Adult mosquitoes were collected in the Galápagos Islands with miniature UV light traps or with miniature incandescent light traps with photoswitch-controlled CO_2 release system (John W. Hock Company). Samples were brought back to the Galápagos Genetics, Epidemiology and Pathology Laboratory to separate the mosquitoes from other insects collected, identify the mosquito species by using morphological features, and store them at −20 °C. Some mosquito specimens were collected as larvae from oviposition traps, reared to adulthood, then stored at −20 °C.

Phylogenetic Study: Molecular Methods. The abdomen was removed from female mosquito specimens before extraction and the whole body was used with male specimens. DNA was extracted by using a salting-out extraction method (31). For the mtDNA sequence analysis, we amplified a portion of the mitochondrial gene *COII* by PCR using primers and protocol from ref. 32, with reverse primer modified as 5′-GATTTAAGAGATCATTACTTGC-3′; and we amplified a portion of the *ND5* gene by using primers and protocol from ref 33. PCRs were performed in 30 μL volume with 0.5 μM of each primer and 1.5 to 3.5 mM $MgCl_2$. PCR products were purified with QiaQuick kit (Qiagen) following the manufacturer's instructions. Purified products were sequenced in both directions with an ABI Automated Sequencer (Applied Biosystems) at the sequencing facilities provided at the University of Leeds and Sheffield, U.K. Samples defining new haplotypes were sequenced twice to be sure they were not PCR artefacts. For the microsatellite genotyping, we used 12 microsatellite markers described by Bataille et al. (34) and followed the methods of amplification and genotyping used by those authors.

mtDNA Phylogenetic Analysis. Sequences of 654 bp (*COII*) and 588 bp (*ND5*) were aligned using ClustalW (35) as implemented in BioEdit software (36). Because both markers displayed a low level of variation, the 2 markers were combined (1,242 bp) to increase the resolution power of the data analysis. We considered the combined markers as a single dataset for the phylogenetic analysis because both datasets (*COII* and *ND5*) were characterized by the same nucleotide frequencies and the best-fit nucleotide substitution model found with MODELGENERATOR for the 2 datasets was identical (HKY + I). Phylogenetic relationships between haplotypes were inferred by using a maximum likelihood approach as implemented in TreeFinder (37) with a bootstrap analysis of 5,000 full bootstrap replicates to test the robustness of the topology. A Bayesian inference approach was also taken by using MrBayes v.3.1.1 (38). Multiple simulations were run for 10 million generations with the first 200,000 discarded as burn-in period after confirming the convergence of chains. Trees were sampled every 1,000 generations and a 50% consensus tree was constructed from the results. No suitable outgroups were found for the inference of *Aedes taeniorhynchus* intraspecific phylogeny. Therefore, we performed the analysis without outgroups and constructed unrooted trees. Haplotype and nucleotide diversity, tests for polymorphism, and mismatch distribution analysis were calculated by using DnaSP v.4.10 (39). A median-joining network was constructed with the same combined sequence dataset using the program NETWORK v.4.2 (http://www.flux-engineering.com) to infer the relationships between the haplotype.

Microsatellite Data Analysis. Heterozygosity values and frequency of null alleles were estimated by using the program CERVUS (40). Conformity to Hardy–Weinberg equilibrium and linkage disequilibrium were determined with GENEPOP 4.0 (41). Summary statistics for the 12 microsatellite loci are presented in Table S4. Pairwise F_{ST} values between populations and their significance (10,000 permutations) were calculated in ARLEQUIN v2.0 (42). The proportion of shared alleles (43) and Cavalli-Sforza genetic distance (44) between populations were calculated with MSA v.4.05 (45) by using a bootstrap analysis of 50,000 replications. The distance matrices obtained were used to construct 50% consensus trees with the neighbor-joining method implemented in PHYLIP v.3.68 (46). Bayesian individual clustering was performed in the software INSTRUCT (47), with the admixture model assuming a number of cluster from $K = 1$ through $K = 18$ for the whole dataset, and then for the Galápagos dataset alone. The program was run 3 times for each value of K for 300,000 generations with 100,000 burn-in steps, and the most likely K was identified by using the deviance information criteria. Population clustering was performed in BAPS v5.1 (48). We used the program GeneClass2 (49) to identify potential migrants, with probability values calculated from a Monte Carlo resampling of 10,000 simulations.

Divergence Time Estimates. The time since most recent common ancestor between Galápagos and continental haplotypes was calculated with a Bayesian approach by using the program BEAST (50) on our mtDNA dataset. A likelihood ratio test, as implemented on DAMBE (51), rejected the strict molecular clock hypothesis for our data. Therefore, we used a relaxed molecular clock (52) with a mean substitution rate of 1.15% per million years for the Bayesian estimation, which is a generalized estimate for the early rate of divergence of insect mtDNA genes (20). We ran the simulations for 6 million generations under different coalescent prior settings, sampling every 1,000 generations, and the settings characterized by the best Bayesian factor value (53) were chosen. The upper and lower 95% higher-posterior-density distribution values were used as confidence interval limits for the estimate.

Bloodmeal Analysis. DNA was extracted from the abdomen of bloodfed *Aedes taeniorhynchus* caught on various islands by using a standard phenol/chloroform extraction method. A portion of the *cytb* gene was amplified by PCR following a protocol described in ref. 29, and purified products were sequenced by using an ABI 3730 Automated Sequencer (Applied Biosystems) at Core Genetics Services, University of Sheffield, U.K. Sequences were compared with sequences available in the GenBank database to identify the species on which each mosquito had fed.

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